Asian Journal of Chemistry; Vol. 24, No. 9 (2012), 3821-3824

ASIAN JOURNAL OF CHEMISTRY

www.asianjournalofchemistry.co.in

Study on Identification and Separation of the Antifungal Antibiotic from its Fermentation Broth of *Streptomyces hygroscopicus* BOS-013 Strain

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(*Received*: 20 May 2011; *Accepted*: 8 April 2012) AJC-11247

BOS-013 actinomycete strain was obtained by separating difference soil of areas from Chang Bai Mountain. The antifungal antibiotic produced by *Streptomyces hygroscopicus* BOS-013 was purified by means of macro-porous adsorbent resin and thin-layer chromatography. The separation and purification of the antifungal antibiotic from its fermentation broth of *Streptomyces hygroscopicus* BOS-013 strain by simulated moving bed chromatography and the crystal of the antibiotic with high purity was obtained. In this paper, the methods of purification by adsorbing of microporous adsorbent resin and detection by high performance liquid chromatography with mass spectrum (HPLC-MS) were established. The study lays a good foundation for its physical-chemical properties. Meanwhile,we identified its structure by spectral analyses. Quasi-molecular ion peak $[M + H]^+$ was 547 given by positive ion mode-electrospray ionization-mass spectrometry and pushed-out its molecular weight was 546. Its molecular formula was $C_{30}H_{43}O_9$. We did not find this compound, so it was infered as a new compound temporarily after Scifinder search.

Key Words: Streptomyces, Screen, Antibiotic, Isolation and purification, Identification.

INTRODUCTION

Actinomycetes, a kind of mycelium-like growth prokaryotes were mainly propagated by spore and have strong terrestrial habits. *Actinomycetes* are of many types and have different metabolic function, it's a kind of practical use with a wide range of biological resources. In the antibacterial, they have about 70 % from *actinomycetes*[1,2]. Antibiotic drug have acquired lot of attention by the world, which are pest control agents of bactericidal function, characteristics of organisms with life and metabolic secretions, replicate. It has many functions, such as low toxicity, efficient, selectivity, *etc*. In this paper, we collected different soil types in Changbai Mountain in China which were isolated *actinomycetes* and purified antibacterial activity components obtained from streptomyces BOS-013 and preliminary identification of its molecular structure was determined. The results were further explored on the basis of its mechanism of action.

EXPERIMENTAL

Source strain: Streptomyces strains BOS-013 and plant pathogenic fungi for biological detection.

Culture medium: Seed medium: Gauze,s Medium No. 1. Bioassay medium: PDA medium. Based fermentation medium: glucose 9 g, corn flour 9 g, soybean meal 9 g, yeast extract 1.2 g, beef extact 0.3 g, $(NH_4)_2SO_4$ 1.2 g, NaCl 0.6 g, K_2HPO_4 0.12 g, $FeSO_4$ 0.006 g, $ZnSO_4$ 0.003 g, $MgSO_4$ 0.06 g, distilled water 300 mL, pH 7.4-7.6.

Optical microscope, electron microscopy, MyCycler PCR, nucleic acid electrophoresis apparatus, protein electrophoresis apparatus, genetic analyzer DNA, BigDye Terminator V3.1 Cycle Sequencing Kit, high-speed centrifuge, constant temperature shaker, three-zones simulated moving bed, freeze dryer, rotary evaporator. Reagents: acetone, ethanol, methanol, Na_2HPO_4, glucose, agar, HCl, NaOH, NaCl, petroleum ether, ethyl acetate, *etc*.

Methods

BOS-013 actinomycete fermentation culture and preparation of sterile culture filtrate: First, the seed fermentation medium were shaken at 28 ºC (100 rpm) *ca.* 5d, then added 1 mL seed solution in based fermentation medium, cultured at 28 ºC (150 rpm) 7 d after, 4000 rpm centrifugal 20 min, spared the supernatant.

Pretreatment of the BOS-013 fermentation broth: Broth with oxalic acid adjusted to pH = 4, standing 0.5 h, 4000 rpm centrifugation to remove Ca^{2+} and Mg^{2+}, then remove the Fe^{3+} by $K_4Fe(CN)_6$ then adjusted to pH = 2 by 1 mol/L HCl, incubated at 60 ºC for 2 h, callback to the original pH value of the level of fermentation broth.

Then, perpared volume ratio is 3:1 with 60 % ethanol and fermentation broth solution and a white flocculent precipitate appeared, centrifugation to remove the resulting hybrid protein precipitation[3].

Extraction of antibacterial substances: Macroporous resin column filled with 10 cm × 100 cm glass column, will be the pre-fermentation liquid BOS-013 the sample of *ca.* 150 mL, then eluted resin column with different concentrations of ethanol solution. Fractions collected tube with 10 mL, then antimicrobial activity were detected, combined active fractions, combined liquid and freeze-dried concentrated rotary steam, obtained coarse crystalline[3,4].

Purification of antibacterial substances: The crude crystals obtained as the simulated moving bed separation of raw materials, then separated and purificated the crude products with self-made simulated moving bed. The separation parameters was separation column (ODS 10 μm, ID. 200 mm × 10 mm), operating mode: 1-1-2, sample concentration: 10 mg/mL, U_F = 0.2 mL/min, U_D = 1.5 mL/min, U_P = 2.5 mL/min, t_s = 12 min, eluent and extrac: methanol with water (v/v) = 12:88 substances with antibacterial activity outflow from the extraction port, impurities out of the raffinate port. Vacuum freeze-drying product solution and collection of products, then to structural analysis.

Preliminary analysis of the structure of antibacterial activity: The purity of samples with HPLC (HPLC-MS, Agilent company). Samples were dissolved in methanol, adjust the concentration of 1×10^3 μg/mL[5,6]. Chromatographic conditions were: Eclipse plus C_{18}, 150 mm × 4.6 mm, the mobile phase was methanol, flow rate is 0.8 mL/min, the temperature of column is 30 ºC, detector is DAD, wavelength is 232 nm. MS conditions were: ion source is APCI, positive, drying temperature is 325 ºC, ion source temperature is 400 ºC, atomization air pressure is 60.0 psi, drying gas flow rate is 5.0 L/min, scan[7].

RESULTS AND DISCUSSION

Research about macroporous resin adsorpte actively antibacterial substances: Select the non-polar CAD-45 macroporous resin as adsorbent. It suits for the separation of antibiotics and has a border adsorbent in polar. For analytical agent, ethanol is the best choice. The active ingredient of its adsorpition and the capacity of antibacterical can be seen in Table-1. The activity of the antibacterial component is high when use 30 % ethanol as eluent and the result has a significant difference with the control. Therefore 30 % ethanol can be selected as a resolution agent.

TABLE-1
RESULTS OF SEPARATING ANTIMICROBIAL SUBSTANES FROM ACTINOMYCETES STRAIN BOS-013 BY MACROPOROUS ADSORPTION RESIN

Number	Solvent		Inhibitory diameter (mm)
	Kinds	Concentration (%)	
1	Ethanol	70	14.7Bc
2	Ethanol	50	24.7ABb
3	Ethanol	30	30.0Aa
4	Control	0	31.0Aa

Eluate the macroporous resin colum with 30 % ethanol, segment, collect and measure the antibicterial activity of the leuent the resulats are shown in Fig. 1. Before 200 mL and after 600 mL there is no antibicterial activity in the collected eluent. The diameter of indicato bactria's inhibition zone *ca.* 9-17 units colleced eluent are 25 mm or more. It has a good separation effect on the elution of this section and concentrate to purification. Other parts of the eluent also isolate a part of the active compounds,it can be thrown away.

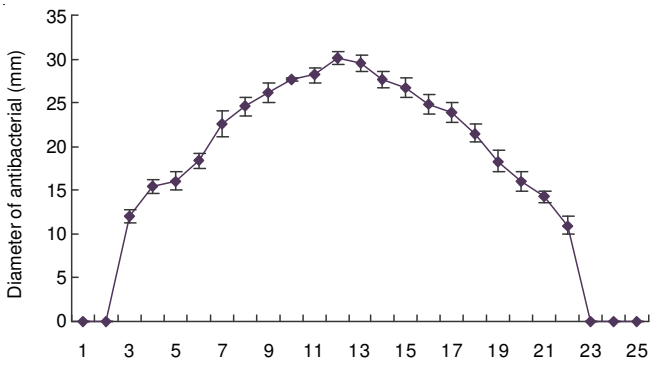


Fig. 1. Adsorption and elution results of actinomycetes strain BOS-013 by macroporous resin

Crude extracts of the actively antibacterial substances of the results of simulated moving bed separation: Separate the crude extracts of macropous resin by using three self-made device with a simulated moving bed and investigate the percentage conent of the final product quality and yield od the product. After identifing the products by the optical, the quality percentage is more than 98 %, the yield is 45 %. The HPLC is shown in Fig. 2 and it is the result of the products extracted by simulated moving bed.

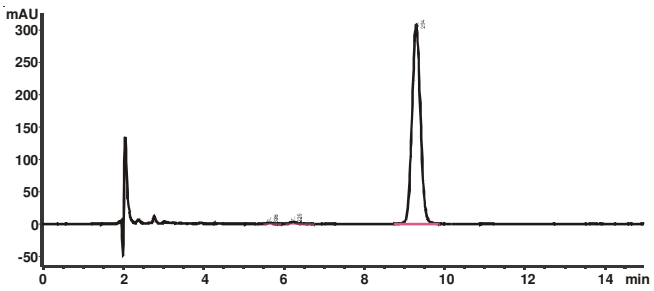
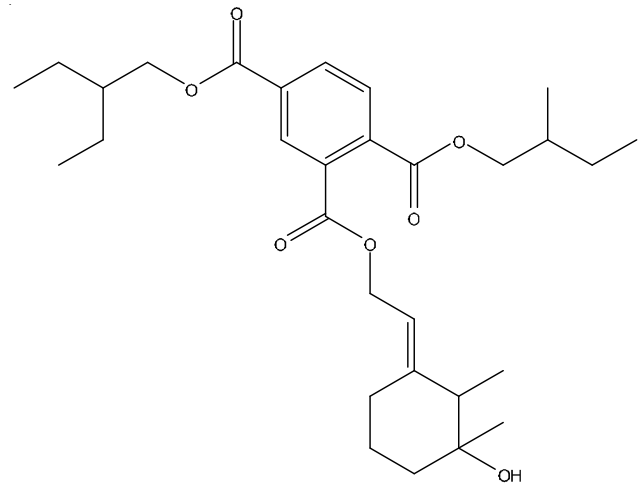


Fig. 2. Chromatography of product in extraction by SMB

Preliminary analysis of the structure of antibacterial substances: The antibacterial substances can be called BOS-013-II which is separated and purified by the simulated moving bed. Its struture is analyzed by UV, IR, HR-MS, ^{1}H-^{1}H COSY, HMBC and other means of spectral and the results of its preliminary characterization are shown in Figs. 3-12. According to the positive ion mode-electrospray ionization-mass spectrometry the excimer ion is $[M + H]^+$ 547. Combined with nuclear magnetic resonance spectroscopy, the molecular formula is $C_{30}H_{43}O_9$, n = 9. And then the specific structure is:



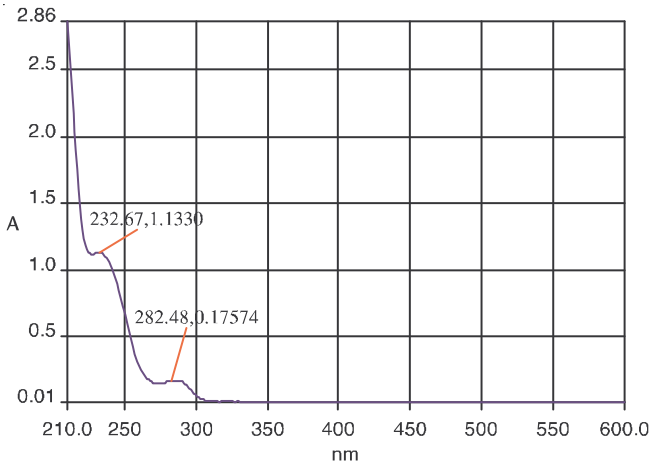


Fig. 3. UV absorb chromatogram map of antimicrobial substances BOS-013-II

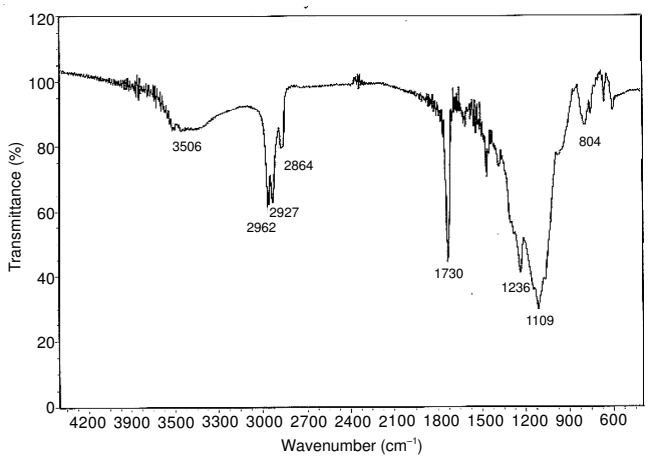


Fig. 4. IR chromatogram of antimicrobial substances BOS-013-II

Conclusion

By *Actinomycetes* fermentation filtrate BOS-013 through macroporous resin, 30 % ethanol elution, vacuum concentration, freeze drying and obtained crude active substance. Methanol and water as the eluent, separation and purification of the crude with self-made three zones simulated moving bed unit. Comprehensive use UV, IR, HPLC-MS, NMR, to preliminary characterization of the structural of BOS-013-II. MS excimer ions was 547 $[M + H]^+$, molecular weight is 546,

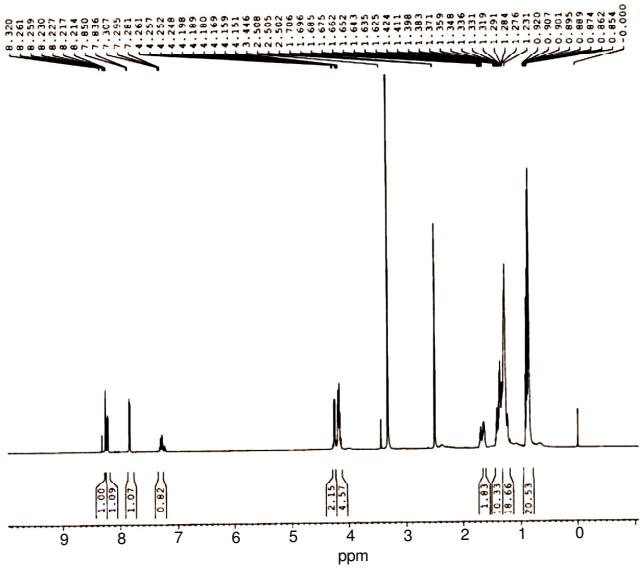


Fig. 5. ^{1}H spectrum of antibacterial substances BOS-013-II

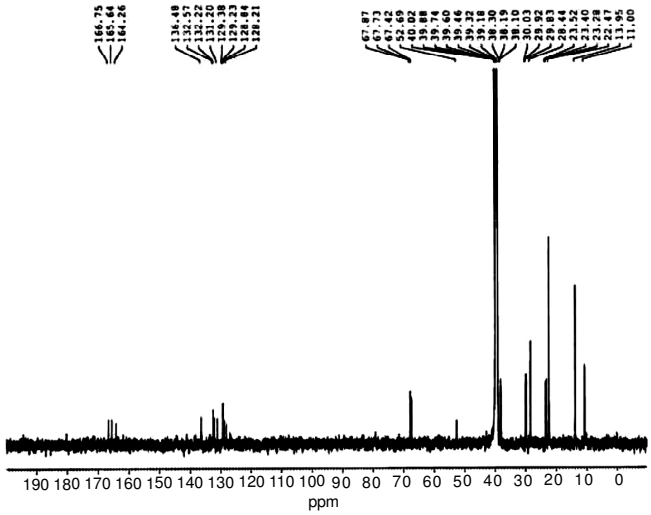


Fig. 6. ^{13}C spectrum of antibacterial substances BOS-013-II

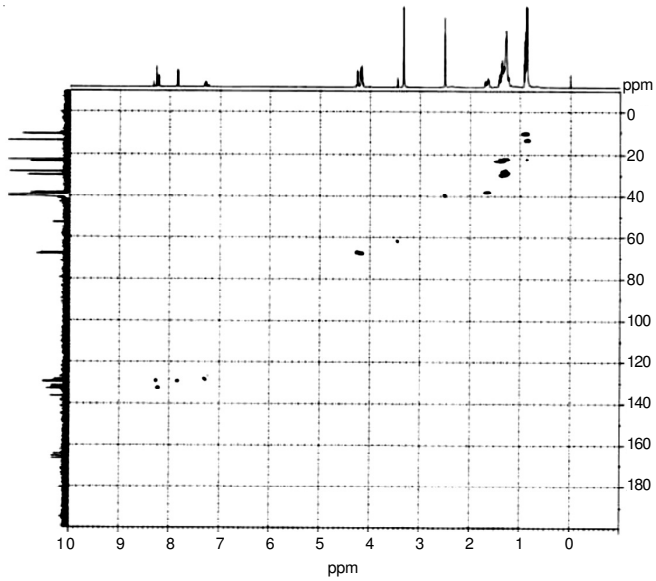


Fig. 7. HSQC spectrum of antibacterial substances BOS-013-II

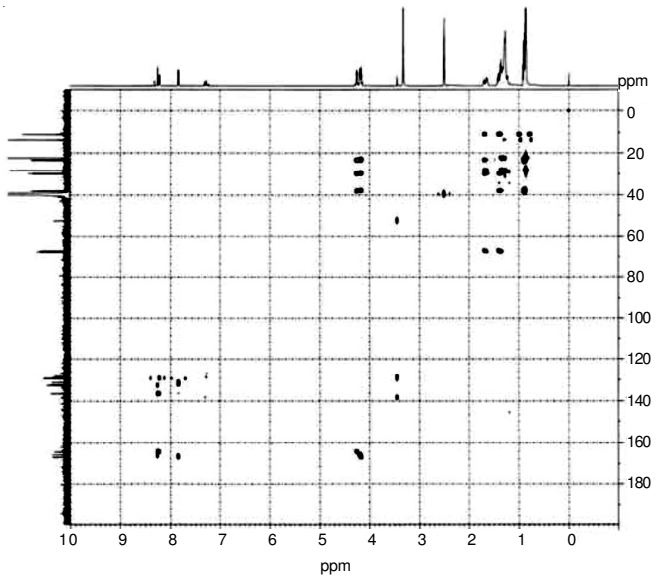


Fig. 8. HMBC spectrum of antibacterial substances BOS-013-II

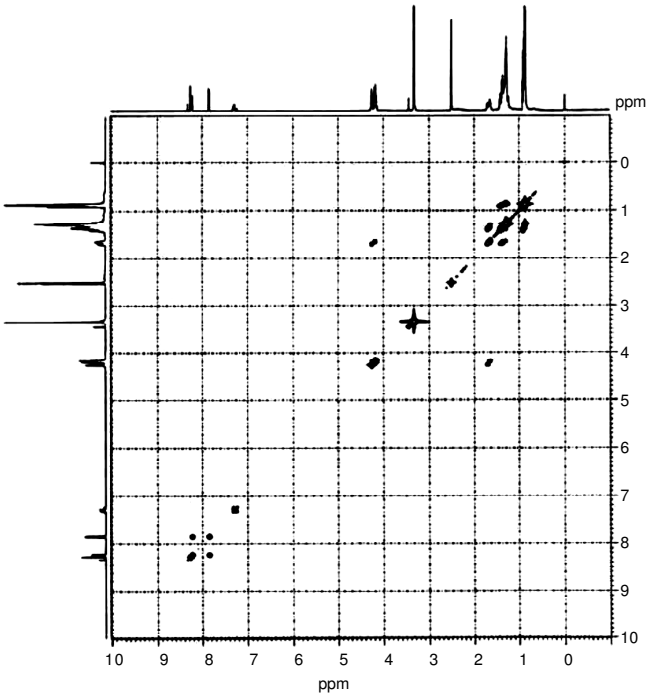


Fig. 9. ^{1}H-^{1}H COSY spectrum of antibacterial substances BOS-013-II

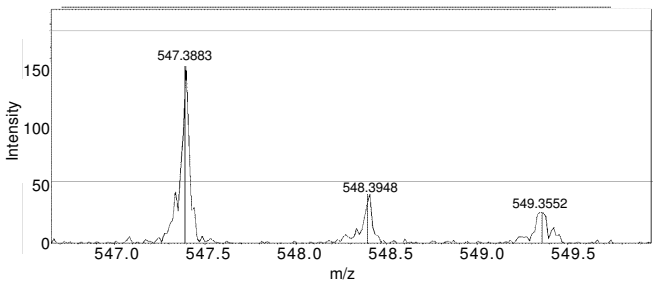


Fig. 10. ESI-MS $[M + H]^+$ spectrum of antibacterial substances BOS-013-II

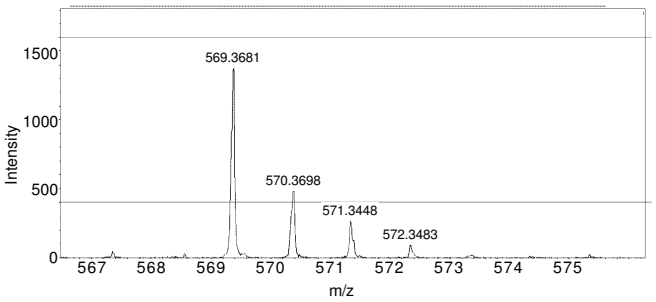


Fig. 11. ESI-MS $[M + Na]^+$ spectrum of antibacterial substances BOS-013-II

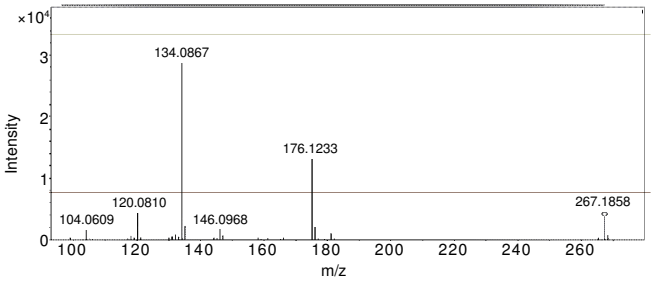


Fig. 12. ESI-MSMS spectrum of antibacterial substances BOS-013-II

combination of nuclear magnetic resonance spectroscopy, determine the formula is $C_{30}H_{43}O_9$. After scifinder search, the compound was not found. So named is [4-(2-ethylbutyl)2-(E)-2-(3-hydroxy-2,3-dimethylcyclohexylidene)ethyl1-(2-methylbutyl)benzene-1,2,4-tricarboxylate].

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